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PLANT PATHOLOGY & NEMATOLOGY - NOTE

Sequence Characterization of Race 4-like Isolates of *Fusarium oxysporum* from Alabama and Mississippi

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ABSTRACT

In 2009 and 2010, isolates of *Fusarium oxysporum* with translation elongation factor sequences (*EF-1α*) identical to those of *F. oxysporum* f. sp. *vasinfectum* race 4 were found on wilted cotton plants in Alabama and Mississippi. Unlike other races of *Fusarium oxysporum* f. sp. *vasinfectum* in the United States, race 4 can cause significant damage to Upland cultivars in the absence of root-knot nematodes; therefore, the discovery of this race in the Southeast could have serious implications. Four of the race 4-like isolates, collected in 2009 and 2010 from Auburn University's E. V. Smith Research Center in Alabama and Mississippi State University's R. R. Foil Research Center, were examined further using sequences of the nuclear ribosomal DNA intergenic spacer region (IGS rDNA) in addition to the translation elongation factor. The four southeastern isolates were identical to reference isolates of race 4 in *EF-1α* sequence, but differed from each other and reference isolates of race 4 in IGS sequence. These results show that *EF-1α* sequence data alone cannot be used to identify race 4 of *F. oxysporum* f. sp. *vasinfectum*, and that the isolates from Alabama and Mississippi are distinct from race 4. Therefore, race 4 of *F. oxysporum* f. sp. *vasinfectum* has not yet been found in the southeastern U.S.

Recent survey efforts in the U.S. have uncovered novel genotypes and new distributions of known races of *Fusarium oxysporum* f. sp. *vasinfectum* W.C. Snyder & H.N. Hansen, the causal agent of Fusarium wilt of cotton (*Gossypium* spp. L.) (Bennett et al., 2011; Castillo et al., 2010; Holmes et al., 2009). Although new virulent genotypes were found in Arkansas and Georgia (Holmes et al., 2009), the discovery of race 4-like isolates of *F. oxysporum* from wilted cotton in Alabama and Mississippi was perhaps the cause for greatest concern (Bennett et al., 2011; Castillo et al., 2010). Prior to its discovery in California in 2001 (Kim et al., 2005), race 4 was found only in Asia (Armstrong and Armstrong, 1960; Assigbetse et al., 1994; Fernandez et al., 1994; Skovgaard et al., 2001). Race 4 has since spread throughout most cotton production areas in the San Joaquin Valley. Complete crop losses from race 4 have been observed in fields planted to highly susceptible Pima cultivars (*G. barbadense* L.) (Davis et al., 2006), and Fusarium wilt caused by race 4 is now the main disease concern for California growers.

The threat posed by *F. oxysporum* f. sp. *vasinfectum* race 4 extends to other areas of the U.S., which mostly produce Upland cotton (*G. hirsutum* L.). Upland cultivars are moderately susceptible to race 4, but severe losses can be sustained in fields with high levels of inoculum (Bennett, unpublished data). Although highly resistant Pima cultivars are available, Upland germplasm with comparably high levels of resistance to race 4 has not yet been identified or developed. In addition, race 4, unlike races 1 and 2 (DeVay et al., 1997), does not require the presence of root-knot nematodes (*Meloidogyne incognita* (Kofoid & White) Chitwood) to cause severe disease (Kim et al., 2005). Fusarium wilt caused by race 4 is observed in both sandy and clay soils, and management strategies for reducing nematode populations will likely be of limited value for managing race 4.

Differential cultivars are traditionally used to identify pathogenic races, but a panel of cultivars capable of separating known *F. oxysporum* f. sp. *vasinfectum* races is not available (Davis et al., 1996). However, multigene genealogies were able to separate races of *F. oxysporum* f. sp. *vasinfectum* into five lineages, and races 4 and 7 were placed in Lineage IV (Kim et al., 2005; Skovgaard et al., 2001).

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Race 7, originally identified though pathogenicity assays on cotton and non-cotton hosts (Chen et al. 1985; Davis et al. 2006), appears to be genetically identical to race 4. Races 4 and 7 share RAPD and rDNA-RFLP profiles, belong to the same vegetative compatibility group, and were identical in pathogenicity on one panel of cotton cultivars (Assigbetse et al. 1994; Fernandez et al. 1994; Kim et al. 2005).

The isolates from wilted cotton plants in Alabama and Mississippi were tentatively identified as *F. oxysporum* f. sp. *vasinfectum* race 4 from sequences of the nuclear rDNA internal transcribed spacer region (ITS; Castillo et al., 2010) and translation elongation factor gene (*EF-1α*; Bennett et al., 2011). Because two divergent copies of ITS were found in *Fusarium* (O'Donnell and Cigelnik, 1997), this region is generally not used to infer evolutionary relationships in this genus. In contrast, *EF-1α* was the most informative gene used in previous studies of *F. oxysporum* f. sp. *vasinfectum* (Kim et al., 2005; Skovgaard et al., 2001), and is widely used for *Fusarium* phylogenetics and diagnostics (Baayen et al., 2000; Geiser et al., 2004; Zhang et al., 2006). *EF-1α* also identified new genotypes of *F. oxysporum* f. sp. *vasinfectum* from 61 isolates collected from the southeastern U.S. (Holmes et al., 2009). Recently, the highly variable nuclear rDNA intergenic spacer region (IGS) was used in combination with *EF-1α* to separate 850 isolates of *F. oxysporum* into 256 two-locus haplotypes (O'Donnell et al., 2009). These haplotypes, or sequence types (STs), had unique sequences for the combined *EF-1α* and IGS datasets. This large study identified 23 unique two-locus STs among 134 isolates of forma specialis *vasinfectum* and one *F. oxysporum* isolate from a *Gossypium* sp. While some lineages of *F. oxysporum* f. sp. *vasinfectum* were split into multiple STs, all isolates in Lineage IV (races 4 and 7) were in ST 31. In addition, ST 31 included only Lineage IV isolates, and did not contain other *F. oxysporum* or races of forma specialis *vasinfectum* (O'Donnell et al., 2009). Both loci were needed to identify Lineage IV as a unique ST. ST 31 was identical in sequence to eight other STs at the *EF-1α* locus and three STs at the IGS locus. However, none of the isolates in the STs with sequences identical to race 4 were forma specialis *vasinfectum* or were isolated from *Gossypium* (O'Donnell et al., 2009). The goal of this study was to further characterize the race 4-like isolates from Alabama and Mississippi with IGS rDNA sequence data in addition to *EF-1α*.

MATERIALS AND METHODS

Five isolates with morphology typical of *F. oxysporum* were obtained from cotton plants showing symptoms of Fusarium wilt in Alabama and Mississippi (Table 1). The isolates from Alabama were collected in 2009 and 2010 from cotton breeding plots of Auburn University's E. V. Smith Research Center in Milstead. The two isolates from Mississippi originated from diseased plants collected in 2009 from the University's R.R. Foil Research Center in Mississippi State. Both fields were infested with root-knot nematodes. Single-spore cultures were made of each isolate as described previously (Bennett et al., 2008).

Isolates were grown on 4 x 4-cm pieces of sterile cellophane placed on the surface of ¼-strength potato dextrose agar (BD Difco, Franklin Lakes, NJ). After four days of incubation in the dark, mycelium was harvested from the cellophane and lyophilized overnight. DNA was obtained from lyophilized mycelium with the FastDNA Kit and FastPrep Instrument (QBiogene, Irvine, CA) following manufacturer protocols. Partial sequences of *EF-1*α and IGS rDNA were amplified in final volumes of 20 µl, containing 5-20 ng of genomic DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, and 0.25 units of GoTaq DNA polymerase (Promega, Madison, WI). Previously described PCR primers (*EF-1*α, EF-1 and EF-2; IGS rDNA, NL11 and CNS1) and thermocycler conditions (O'Donnell et al., 2009) were used. PCR products were visualized on 1.5% agarose gels stained with SYBR-Safe (Invitrogen, Carlsbad, CA). Gel fragments containing bands of expected size for *EF-1*α were excised with a clean scalpel, dissolved in 200 µl of 5.5M guanidine thiocyanate at 50°C, and purified through silica membrane tubes (Epoch Life Science, Missouri City, TX). Purified *EF-1*α DNA was eluted from the silica membranes with 15 µl of 10 mM TRIS buffer. IGS rDNA PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA). The IGS rDNA (primers U46.67, RU46, iNL11, RU3, IGSF4, iCNS1, CNSa, and NLa; Mbofung et al., 2007; O'Donnell et al., 2009) and *EF-1*α (primers EF-3 and EF-22; O'Donnell et al., 1998, 2009) were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA). Extension products were purified using the ethanol-EDTA precipitation protocol of the sequencing kit, and samples were run on an ABI 3130 capillary sequencer.

Table 1. Isolates of *Fusarium oxysporum* used in the study.

Isolate [z]	ST [y]	*F. oxysporum* forma specialis [x]	Races [w]	Host / Substrate	Origin	*EF-1α* Genbank Number	IGS Genbank Number
AL-KL11	-	-	-	*Gossypium hirsutum*	AL-USA	KC549662	KC549658
AL-KL25	-	-	-	*G. hirsutum*	AL-USA	KC549663	KC549659
MS-GL10	-	-	-	*G. hirsutum*	MS-USA	KC549664	KC549660
MS-GL18	-	-	-	*G. hirsutum*	MS-USA	KC549665	KC549661
NRRL 22557	23	*vasinfectum*	-	*Gossypium* sp.	Unknown	FJ985276	FJ985467
NRRL 25420	28	*vasinfectum*	1, 2	*Gossypium* sp.	USA	AF008512	FJ985472
NRRL 25424	29	*vasinfectum*	2	*G. hirsutum*	CA-USA	FJ985277	FJ985473
NRRL 25429	30	*vasinfectum*	3, 5	*G. hirsutum*	Egypt	FJ985278	FJ985474
NRRL 25434	31	*vasinfectum*	4, 7	*Gossypium* sp.	India	FJ985279	FJ985475
NRRL 25437	32	*vasinfectum*	6	*Gossypium* sp.	Brazil	FJ985280	FJ985476
NRRL 26406	63	*melonis*	3	*Cucumis melo*	Mexico	AF008504	AB106056
NRRL 26677	81	-	1, 8	Human	Australia	AY527528	AY527725
NRRL 31495	113	*vasinfectum*	3	*Gossypium* sp.	CA-USA	FJ985306	FJ985539
NRRL 32558	115	*vasinfectum*	01111	*Gossypium* sp.	Australia	FJ985308	FJ985541
NRRL 32562	116	*vasinfectum*	01112	*Gossypium* sp.	Australia	FJ985309	FJ985542
NRRL 32873	117	*vasinfectum*	-	*Gossypium* sp.	AR-USA	FJ985310	FJ985543
NRRL 32881	118	*vasinfectum*	108	*Gossypium* sp.	GA-USA	FJ985311	FJ985544
NRRL 32882	119	*vasinfectum*	-	*Gossypium* sp.	AR-USA	FJ985312	FJ985545
NRRL 32883	120	*vasinfectum*	110	*Gossypium* sp.	AR-USA	FJ985313	FJ985546
NRRL 32885	121	*vasinfectum*	112	*Gossypium* sp.	AR-USA	FJ985314	FJ985547
NRRL 32887	122	*vasinfectum*	-	*Gossypium* sp.	LA-USA	FJ985315	FJ985548
NRRL 32890	123	*vasinfectum*	-	*Gossypium* sp.	AR-USA	FJ985316	FJ985549
NRRL 32891	124	*vasinfectum*	140	*Gossypium* sp.	AR-USA	FJ985317	FJ985550
NRRL 32897	125	*vasinfectum*	127	*Gossypium* sp.	AR-USA	FJ985318	FJ985551
NRRL 34079	133	*vasinfectum*	-	*Gossypium* sp.	LA-USA	FJ985323	FJ985556
NRRL 36092	134	-	-	*Gossypium* sp.	Unknown	FJ985324	FJ985557
NRRL 38542	220	*vasinfectum*	3	*G. barbadense*	Israel	FJ985407	FJ985642
FOV14	31	*vasinfectum*	4	*Gossypium* sp.	CA-USA	DQ837695	DQ831885
NRRL 22518	3	*melonis*		*Cucumis melo*	MI-USA	FJ985265	FJ985447
NRRL 25375	26	-	-	Human	South Pacific	AY527521	FJ985470
NRRL 26221	45	*cucurbitacearum*	-	*Cucumis sativus*	The Netherlands	FJ985283	FJ985489
NRRL 25387	27	-	-	Human	New Zealand	AY527527	FJ985471
NRRL 26413	67	*momordicae*	-	*Momordica charantia*	Taiwan	FJ985291	FJ985498
NRRL 26444	74	*melongenae*	-	*Solanum melongena*	SC-USA	FJ985297	FJ985505
NRRL 26447	76	*sesami*	-	*Sesamum* sp.	SC-USA	FJ985299	FJ985507
NRRL 26679	82	-	-	Human	Australia	AY527526	AY527723
NRRL 26874	85	*spinaciae*	-	*Spinacia oleracea*	AR-USA	AF246849	FJ985512
NRRL 26875	86	*spinaciae*	-	*Spinacia oleracea*	AR-USA	AF246850	FJ985513
NRRL 38277	174	*tracheiphilum*	-	Unknown	Unknown	FJ985364	FJ985597
NRRL 38300	184	-	-	Boxwood	VA-USA	FJ985373	FJ985607
NRRL 38303	187	-	-	*Embothrium coccineum*	Chile	FJ985375	FJ985609
NRRL 38445	208	*lycopersici*		*Solanum esculentum*	CA-USA	FJ985395	FJ985630
NRRL 38544	221	-	-	*Fragaria* sp.	New Zealand	FJ985408	FJ985643
NRRL 38555	224	-	-	*Persea americana*	New Zealand	FJ985411	FJ985646
NRRL 38585	225	*perniciosum*	-	*Albizia julibrissin*	Unknown	FJ985412	FJ985647
NRRL 38586	226	*perniciosum*	-	*Albizia julibrissin*	VA-USA	FJ985413	FJ985648
NRRL 38593	227	-	-	*Zea mays*	New Zealand	FJ985414	FJ985649
F. foetens	59	-	-	*Begonia* hybrid	The Netherlands	AY320087	FJ985680
F. foetens	186	-	-	*Pinus radiata*	Chile	FJ985444	FJ985679

[z] NRRL (National Center for Agricultural Utilization Research, Peoria, IL) isolates and *Fusarium foetens* (outgroup) from O'Donnell et al. (2009); FOV14 from Mbofung et al. (2007). Sequences were downloaded from the GenBank website (http://www.ncbi.nlm.nih.gov/genbank).

[y] ST = two-locus sequence type defined by O'Donnell et al. (2009) using IGS rDNA and *EF-1α*.

[x] *Forma specialis* of isolate representing ST, if applicable.

[w] Race or genotype of *F. oxysporum* f. sp. *vasinfectum* belonging to ST, if applicable (Bentley et al., 2000; Holmes et al., 2009; O'Donnell et al., 2009).

EF-1α and IGS rDNA sequences from each representative of the 23 two-locus STs containing f. sp. *vasinfectum* or *F. oxysporum* isolated from *Gossypium* were obtained from GenBank (Table 1; O'Donnell et al., 2009). The online FUSARIUM-ID database (http://www.fusariumdb.org; (Park et al., 2011) was used to identify STs with sequences identical to ST 31 (race 4) at the *EF-1α* (STs 3, 27, 67, 85, 86, 174, 208, 227) and IGS (STs 26, 221, 224) loci. STs identical in IGS sequence to the race 4-like isolates from Alabama and Mississippi were also included. IGS rDNA and *EF-1α* sequences of FOV14, a race 4 isolate of *F. oxysporum* f. sp. *vasinfectum* from California, was also downloaded from Genbank (Mbofung et al., 2007). Sequence data were edited in SeqMan Pro and aligned using the ClustalW algorithm in MegAlign (DNASTAR, Madison, WI). Maximum parsimony analyses were conducted in PAUP v. 4.0b (Sinauer Associates, Sunderland, MA). All characters were unordered and given equal weight, and alignment gaps were considered as missing data. *EF-1α* and IGS rDNA sequences from two isolates of *Fusarium foetens* Schroers, a sister taxon to *F. oxysporum* (Schroers et al., 2004), were used as outgroups (O'Donnell et al., 2009). Maximum parsimony trees were inferred, using the heuristic search option and 1000 random addition sequences with tree bisection-reconnection branch swapping. Support for the internal nodes was measured with 1,000 parsimony bootstrap replications.

RESULTS AND DISCUSSION

The two race 4-like isolates from Alabama, AL-KL1 and AL-KL11, had identical *EF-1α* and IGS sequences, so only one of these isolates (AL-KL11) was included in the analyses. BLAST queries on the FUSARIUM-ID website revealed IGS sequences for isolates AL-KL11 and MS-GL18 were unique. The IGS sequence of isolate AL-KL25 was identical to six STs (74, 82, 184, 187, 225, and 226), and MS-GL10 was identical to ST 45 and ST 76. Therefore, sequences from a total of 49 isolates were analyzed (Table 1). The 633-bp *EF-1α* dataset had 48 polymorphic sites, 37 of which were phylogenetically informative. The 2111-bp IGS rDNA dataset had 187 phylogenetically informative characters among 305 polymorphic sites.

Maximum parsimony analysis of the *EF-1α* data generated a single tree with a length of 51 steps. Tree topology was similar to results from previous analyses in that Lineages III (race 8), IV (race 4), and V (Australian biotypes) of *F. oxysporum* f. sp. *vasinfectum* formed distinct clades (Figure 1; Skovgaard et al., 2001; Kim et al., 2005; Holmes et al., 2009). The race 4-like isolates from Alabama and Mississippi grouped with ST 31 and FOV14, the reference isolates of *F. oxysporum* f. sp. *vasinfectum* race 4, as in previous reports (Castillo et al., 2010; Bennett et al., 2011). However, this clade also included 13 additional STs, including ST 23, which is composed of four isolates of forma specialis *gladioli* and one isolate of *F. oxysporum* f. sp. *vasinfectum* of unknown origin. ST 23 is identical in *EF-1α* to ST 31 except for a gap at position 305 on the alignment. In contrast, ST 31 was in an unresolved branch in the *EF-1α* tree generated from 850 isolates of *F. oxysporum* (O'Donnell et al., 2009). The 850-isolate dataset, treated gaps as an informative character and had 101 phylogenetically informative sites in contrast to the 37 found in this study. The IGS data produced 56 most parsimonious trees (331 steps in length) with significant topological differences from the *EF-1α* tree. In the IGS tree, the race 4-like isolates from Alabama and Mississippi no longer grouped with ST 31 or FOV14. As expected from preliminary BLAST searches, isolate AL-KL25 was in a clade with STs 23, 74, 82, 184, 187, 225 and 226, and MS-GL10 grouped with STs 45 and 76. Isolate MS-GL18 was similar to STs 174 and 86, but AL-KL 11 did not appear to be closely related to other *F.oxysporum* isolates. FOV14 and ST 31 were in a clade with STs 26, 221, and 224, but FOV14 was not identical in IGS sequence to ST 31. O'Donnell et al. (2009; Supplementary Table 1) sequenced FOV9, another race 4 isolate of *F. oxysporum* f. sp. *vasinfectum* from California, but this difference is unexpected. Previous work has shown limited genetic variation among race 4 isolates from California (Yang et al., 2006).

While the race 4-like isolates from Alabama and Mississippi could not be identified to a sequence type of O'Donnell et al. (2009) using this small dataset and simplified analyses, these results clearly indicate that these isolates are not identical to *F. oxysporum* f. sp. *vasinfectum* race 4 from California or Asia. Preliminary pathogenicity assays also supported these results. The Mississippi isolates and AL-KL11 caused mild symptoms on cotton, and AL-KL25 was more virulent on Upland cotton than FOV14 (R. Bennett, unpublished data). Therefore, the confirmed distribution of *F. oxysporum* f. sp. *vasinfectum* race 4 in the U.S. remains limited to California. These results also show that *EF-1α* data alone are insufficient for identifying race 4, and that additional data such as IGS sequences are needed. This information should be useful to future efforts in monitoring the spread of *F. oxysporum* f. sp. *vasinfectum* race 4 in the U.S.

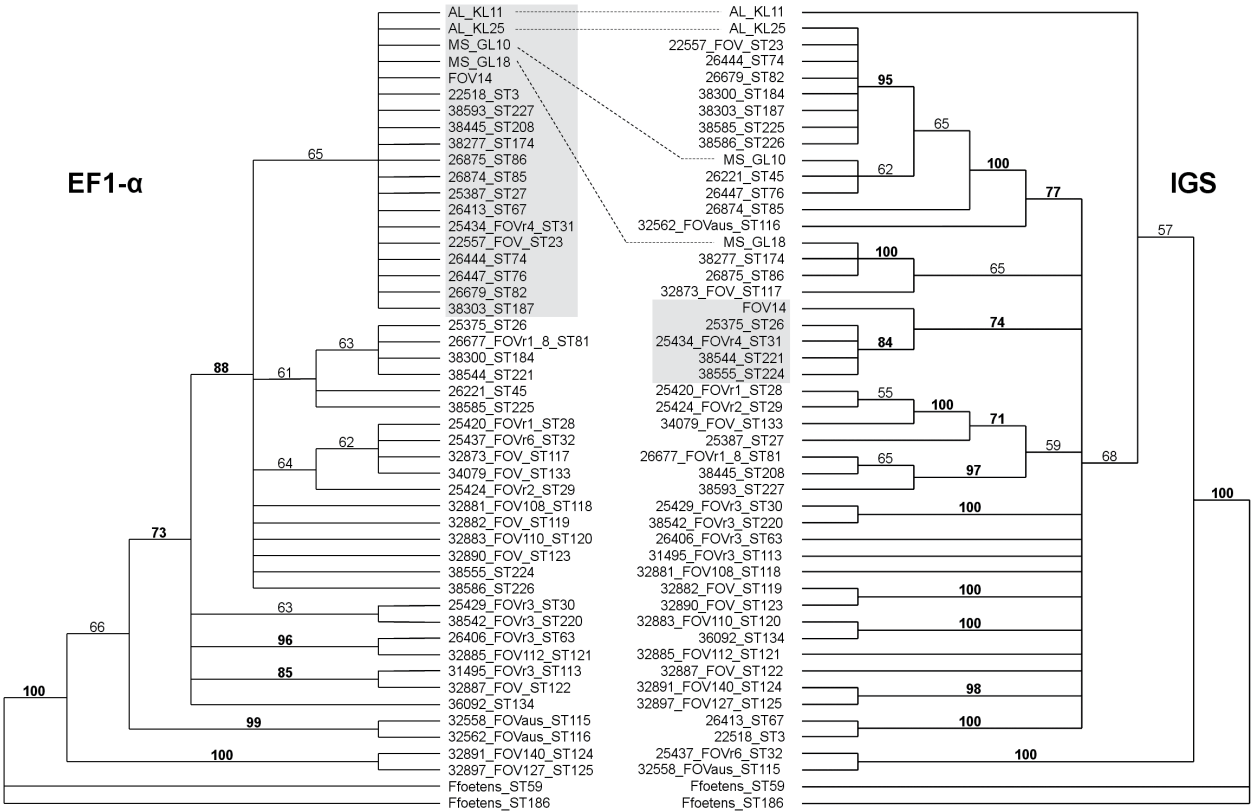

Figure 1. Single most parsimonious tree generated from *EF*-1α data (tree length = 51 steps; consistency index (CI) = 0.96; retention index (RI) = 0.98); and consensus of 56 most parsimonious trees from IGS rDNA data (consensus tree length = 379 steps; CI = 0.53; RI = 0.81). Numbers above nodes indicate bootstrap support from 1000 replicates; values ≥ 70% in bold. Isolates representing sequence types (ST) marked with NRRL number, ST number, and if applicable, race(s) of *Fusarium oxysporum* f. sp. *vasinfectum* included in the ST. Clades including reference isolates of *F. oxysporum* f. sp. *vasinfectum* race 4 highlighted in gray. Dashed lines mark incongruence between trees for race 4-like isolates from Alabama and Mississippi.

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